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PRINCIPAL INVESTIGATOR: Anthony Lee

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

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Anthony Lee

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

E-Mail: anthonyl@mail.med.upenn.edu

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Introduction

The purpose of my project remains the same. In the paper Vieira *et al.* (1996), it was demonstrated that endocytosis of EGF receptors (EGFR) played a role in the regulation of their signaling. EGFR is internalized followed its activation and dimerization with a second receptor. A second receptor that is internalization-impaired, such as erbB-2, would presumably slow down internalization of EGFR. Internalization and down-regulation of receptors is the mechanism by which cells control signaling from EGFR. Inhibition of endocytosis leads to persistence of the activated receptors on cell surface and increases the duration and strength of signaling from EGFR, which may lead to unregulated cell growth and transformation. My plan is to show that erbB-2 does indeed affect EGFR endocytosis, and this will lead to alteration of downstream signaling. By making mutations in the regulatory cytoplasmic tail of erbB-2, I could potentially enhance its capability for endocytosis, and this should restore normal signaling to EGFR.

Body

The first task is to develop an assay to measure internalization of EGF/EGFR. For this I chose the ELISA assay because it is a fast, reliable, and quantitative assay. It is also an assay that the lab of Sandra Schmid had used previously to measure EGF internalization. Unexpectedly, this assay has failed to work for EGF. In my last report, I had found that the assay work for measuring transferrin uptake and was confident that the same will be true for EGF. One problem was that the antibody cited in her work is no longer available. The assay requires a capture antibody to capture biotin-labeled EGF, which is then detected by streptavidin-linked horseradish-peroxidase (HRP). The company that sold the antibody no longer does so. An extensive search for a substitute and testing by trials-and-errors failed to turn up an alternative. I sought to overcome this problem by using a cell-based ELISA assay that does not use a capture antibody. This assay, which was developed by Renate de Wit, uses the cells internalizing the EGF as the de-facto capture antibody. Cells are then fixed and permeabilized and the internalized EGF is detected by streptavidin-HRP. I tested this assay but found that the assay has too high a background, as a result, is not useful as a quantitative assay.

I am now working on another alternative assay. This assay measures tyrosine phosphorylation on a molecule named hrs1, which stands for hepatocyte-growth factor regulated substrate 1. This molecule generally localizes to early endosomes, to which EGF and its receptor are destined following endocytosis. It has been shown by Urbe and co-workers (2000) that tyrosine phosphorylation on hrs1 is dependent on endocytosis and colocalization of EGF/EGFR with hrs1 in early endosomes. In cells in which endocytosis

is blocked, using different method including the use of dominant-negative dynamin, hrs1 phosphorylation is inhibited. My testing of this assay with the same dominant-negative dynamin, K44A, confirmed this observation. In cells overexpressing wild type dynamin, after cells were incubated with EGF at 37 °C (at this temperature cells can internalize EGF), immunoblot of cell lysate immunoprecipitated with hrs1 antibody (a generous gift from Sylvie Urbe) showed robust tyrosine phosphorylation on hrs1. In contrast, in cells incubated with EGF on ice (this blocks EGF internalization) no band corresponding to tyrosine-phosphorylated hrs1 is seen. In cells overexpressing K44A, which inhibited receptor-mediated endocytosis in general, the intensity of the tyrosine-phosphorylated band is substantially reduced. The residual band may reflect the fact that neither expression of K44A nor inhibition of endocytosis is complete in all K44A cells. The usefulness of the assay depends on the inherent expression of hrs1 in the cell line used, which will vary for different cell lines. I expect that introduction of a recombinant HA-tagged hrs1 by stable transfection should make this assay applicable in all cell lines. In addition, this should increase the dynamic range of the assay in cells that do not express a sufficient amount of hrs1. I am in the process of obtaining the HA-tagged hrs1 from the person who had generated the recombinant construct.

Concurrently, I am working on developing Hela cell lines that stably express erbB-2 at different levels. The point is to compare the endocytosis of EGFR in cells overexpressing erbB-2 at different levels, and, should I find a difference, examine signaling of EGFR as a consequence of the different erbB-2 expression. A stably expressing cell line ensures a constant expression of erbB-2 from experiment to experiment, and comparison of cell lines expressing different levels of erbB-2 allows me

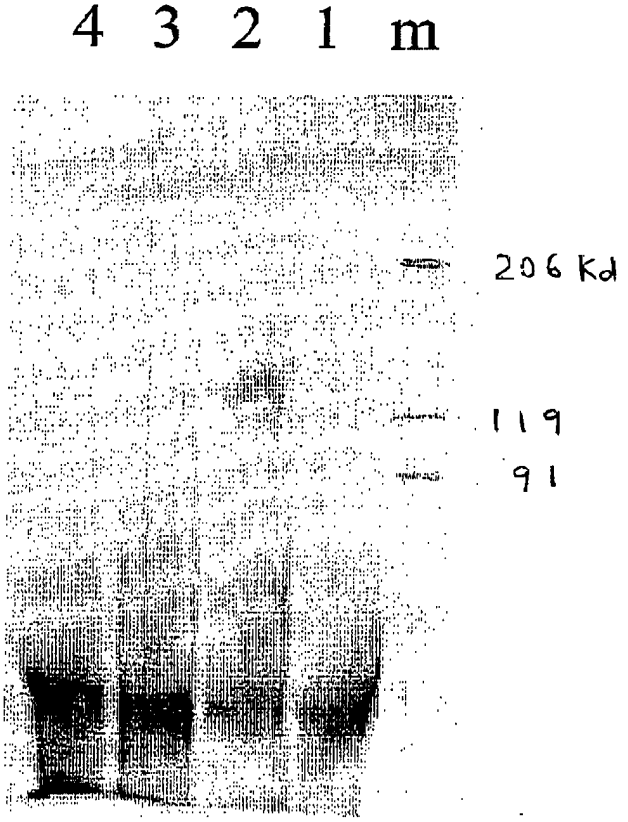
to assess the impact of erbB-2 overexpression on EGFR endocytosis and signaling. Hela cells were chosen because this was the cell line that Vieira and co-workers used. Unexpectedly, I have had much difficulty in generating stable cell lines that express erbB-2 at a substantially higher levels compared to parental line. This is surprising considering that Hela cells are generally considered easy to transfect. The process of selection of stably expressing clone takes about a month, and I have repeated it several times, but at the end I found that the clones that I had screened express only marginally higher level of erbB-2, at best, compared to the parental cells. A second problem is that Hela cells do express a significant amount of erbB-2 endogenously, and it might be unrealistic to expect that I can substantially increase the level of expression with the transfection methods that I have used (fugene, lipofectamine, lipofectin, and calcium phosphate).

I am considering the possibility of using FACS analysis to isolate higher expressing clones. Several people in my lab have used this technique to select stable cell lines, and it is less time-consuming and more efficient than the approach I am currently using, Western blot. Alternately, I am turning to other cell lines. For example, MDA-MB 468, which expresses a huge amount of EGFR, expresses relatively little erbB-2. A second possibility is Baf-3. This cell line express none of the erbB receptor family. As a result, background expression is not an issue. I have generated erbB-2 transfected clones in MDA-MB 468 and preliminary results look promising. I am still selecting clones in Baf3. These cell lines should work just as well as Hela cells in my endocytosis assay and do not have the background expression of erbB-2 that will complicate analysis.

Because of a lack of a satisfactory erbB-2-expressing stable, I have not yet been able to test the central premise of my project, that overexpression of erbB-2 inhibits the

rate of endocytosis of EGFR. I am still working on obtaining a stable in Hela, but I am now focusing my hope on the MDA-MB 468 and Baf3 cells. If the erbB-2 expressing clones in MDA-MB 468 and Baf-3 cells are satisfactory, an endocytosis assay will quickly show whether the premise of my project is correct. Then the next stage will be to replace the cytoplasmic regulatory region of erbB-2 with that of EGFR, in order to test the assumption that this will enhance the internalization rate of erbB-2 and its heterodimerization partner, EGFR. Finally, if I can establish a link between erbB-2 overexpression and EGFR endocytosis, then it will be meaningful to examine the role of erbB-2 endocytosis on the regulation of EGFR signaling.

EGF Internalization Assay Using Hrs 1



m marker
1 WT dynamin-transfected Hela cells, incubated with EGF on ice
2 WT dynamin, EGF at 37 ℃
3 K44A dynamin, EGF on ice
4 K44A dynamin, EGF at 37 ℃

All incubations are for 15 minutes

Key Research Accomplishments

Development of an endocytosis assay for EGF. Two different methods of inhibiting EGF endocytosis- ice treatment and dominant-negative dynamin- demonstrate the usefulness of the assay.

Development of erbB-2-expressing cell lines in MDA-MB 468 and Baf3 (almost done). These cell lines will be used to examine EGFR endocytosis in the context of erbB-2 overexpression and later, EGFR signaling.

Reportable Outcomes

None.

Conclusion

Given my difficulty in developing an endocytosis assay for EGF and later with erbB-2-expressing stable clones, I have not yet been able to test the central premise of the project. The assay is now ready, and I believe I have stable erbB-2-expressing clones in MDA-MB 468, with ones in Baf3 not far behind. If I can confirm that erbB-2 inhibits EGFR internalization, I can then test the hypothesis that modification of erbB-2's endocytic behavior will lead to change in EGFR signaling.

"So What"

ErbB-2 overexpression is found in many forms of human cancers, including breast cancer. EGFR regulates cell growth and proliferation, and when this regulation is not controlled unabated EGFR signaling may lead to cellular transformation. ErbB-2 may play a crucial role in promoting the presence of activated EGFR on cell surface, by forming a heterodimeric complex with EGFR and retaining it on cell surface. If this is true, then study of the mechanism by which this is accomplished can yield important insight in the fight against cancer.

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